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13. ABSTRACT (Maximum 200 Words) Although considerable progress in the understanding of prostate cancer has been made in the last few years, the basic knowledge of the biology of this disease remains elusive. The development of this cancer is related to the male sexual hormone (testosterone) but the actual mechanisms by which testosterone affects the development of this cancer is not known. The prostate gland has at least three different types of cells that contribute to the physiology of the gland: basal, luminal and neuroendocrine cells. It is not totally clear what the relationship is between these different cell types, how testosterone affects them and which one is the target cell in prostate cancer development. We will use new transgenic technology that allows tagging of a particular cell population and following its behavior over the life of the animal. These experiments will be performed in mice because this technology is well developed in these animals and there is a basic knowledge of the rodent prostate. The studies proposed here will clarify some of the basic aspects of the biology of the prostate gland and the process of carcinogenesis in this organ.				
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Introduction

The purpose of this research is to develop Cre/Lox technology to investigate cell lineages in the prostate gland of the mouse. The epithelium of the prostate is known to be formed by at least three cellular subtypes. The luminal cells have a secretory function and are the major component of the prostate epithelium. Between the layer of luminal cells and the basement membrane is another compartment formed by basal cells. This third cellular type is neuroendocrine cells, a minor population of cells interspersed among the luminal cells (Wang et al., 2001).

The luminal cells are believed to be differentiated cells derived from the basal cells. Although they have proliferative activity, this is limited to a few divisions before they become apoptotic. These cells express androgen receptor and have secretory characteristics under the control of androgens. They express specific markers, e.g. Keratin 8 and 18. The basal cells are very small cells expressing Keratins 5 and 14. They are thought to be the proliferative compartment and it is also believed that at least a subpopulation of the basal cells may be stem cells. Neuroendocrine cells are a minor component probably involved in intercellular signaling. They are also important because many tumors express neuroendocrine markers suggesting that either some tumors derive from this particular cell type or differentiate into this phenotype (Hsieh et al., 1992; El-Alfy M et al., 2000; Wang et al., 2001; Bonkhoff, 2001).

This paradigm of tissue kinetics in the prostate is not supported by hard core evidence and has been questioned by several investigators. Furthermore, although the luminal cells appear to be terminal cells, tumors derived from the prostate have all the characteristics of this cell type. Furthermore, no tumors with the phenotypic characteristic of basal cells have been described and only non-neoplastic hyperplasia has been described in this subpopulation (Foster and Bostwick, 1998).

The goal of this project is to develop a system that allows permanent labeling of the different cell subpopulations using Cre/Lox technology (Saue, 1998). The Cre gene was put under the control of Keratin 5 and 14 promoters (markers of basal cells) as well as the probasin promoter (marker for luminal cells). We have also obtained Rosa 26- β gal mice. Rosa 26 is a universal promoter, in this case driving a β gal gene preceded by a floxed stop sequence (Zambrowics et al., 1997). Crossing these animals with Cre expressing mice led to recombination for the Rosa 26 minigene with loss of the stop sequence and expression of the β gal message. Since keratin markers are lost or gained as cells differentiate, by using the keratin promoters driving the Cre gene, we can permanently label cells in a given compartment and these cells will remain labeled regardless of their differentiation status.

In a previous report we had obtained and/or developed the animal models to conduct our experiments. We also provided evidence of the existence of basal/luminal transition cells in the IGF transgenic model. In addition, we did a pilot experiment using not inducible Cre mice to provide proof of concept and solve any technical problems that may not be previously anticipated. One of the problems perceived in the early experiments is that

current techniques designed to detect the enzymatic activity of β gal do not have the definition and sensitivity to detect the activity in the basal cells. Thus, in this period we have worked to develop an immunologic procedure that can be used by either peroxidase (light microscopy) or fluorescent technique. This is particularly important because the fluorescent strategy will allow the use of confocal microscopy.

BODY

Animals:

We developed all the transgenic mice to be used in the project. This is the updated list of animals and the current status for each of them.

K5-Cre:

These animals have been developed using our K5 cassette, originally obtained from Dr. Jose Jorcano (Ciemat, Madrid, Spain). The colony was established in our facility and used routinely in our transgenic experiments (Robles et al., 1996; Rodriguez Puebla et al., 2000). Crosses with Rosa 26 mice have already been performed (see below).

K14-Cre

These animals have been developed in collaboration with Dr. DiGiovanni and the colony was established at our facility. Crosses with Rosa 26 mice have already been performed (see below).

Rosa 26

These animals were obtained from our Core Facility in Houston with permission from Dr. P. Soriano (Zambrowicz BP et al., 1997) and have already been used in crosses with K5-Cre and K14-Cre.

Probasin Cre

There have been substantial delays with these mice. These animals were originally received from the University of Southern California (Wu et al., 2000). The mice were requested before the starting date of the grant, but a year later there were still problems with the MTA. Lawyers from our institution questioned the language of the MTA submitted by USC. Changes introduced by our lawyers needed to be approved not only by USC but also by pertinent officers of the University of Manitoba as that institution holds the original patent on some of the reagents used to develop these mice. Two males were finally received on April 16, 2002 but we were unable to establish a colony because the mice were sterile. We have had to obtain new breeders from the NIH repository because the University of Southern California has turned these mice over to the repository for distribution. Mice were requested in September 2002 and were finally delivered on January 16, 2003 and the colony has now been established.

K5-CreERT²

These mice are similar to K5-Cre but the Cre gene has been replaced by a fusion protein between Cre and a mutated estrogen receptor. These primers were developed at our facility using reagents from Dr. P. Chambon's laboratory (Indra et al., 1999). Three lines

of these transgenics have been established and they are presently being tested (see below).

K14-CrePR

These animals are identical to K5-CreER^{T2} except the transgene is driven by the K14 promoter. They were obtained from P. Chambon's laboratory (Indra et al., 1999). They are presently being crossed with Rosa 26- β gal mice.

K5-IGF

These mice have been developed at Science Park Research Division (SPRD) and are available in our colony. They were described in the original submission of the grant (DiGiovanni et al., 2000).

TRAMP Mice

This is a metastatic model of prostate cancer generated by transgene expression of a T antigen under the control of the probasin promoter. These animals have been received from Dr. N.M. Greenberg (Gingrich1996) and the colony is stabilized in our animal facility.

Key Research Accomplishments

As discussed in the previous report, we are still behind schedule. We underestimated the time required to obtain the animals and, in particular, to stabilize the colony and breed enough mice to perform the experiments. We have also anticipated some potential problems in the pilot experiment. However, at this point we think that most of the problems have been solved and all the mice necessary for these experiments have been developed and the colonies are established.

Breeding in progress (crosses)

This section lists the status of the different crosses proposed for the project:

K14-CrePR X Rosa 26

This cross will produce recombination (activation) of the Rosa 26 β gal gene in basal cells. If these cells differentiate into luminal cells both population should be β gal positive. If the luminal cells are β gal negative they do not originate from the basal cells.

We have set up crosses of these two transgenics in both directions (male K5-CreERT X female Rosa 26 and viceversa). We have obtained 11 males (born 3/23 and 3/28). These mice are ready to be analyzed.

K5-CreER X Rosa 26

This cross serves essentially the same purpose as the K14-CrePR X Rosa 26 but they are being carried out, as discussed in the grant, for two purposes. First to confirm our results with two independent markers of the basal population and also as a technical back up because we did not determine the efficiency of recombination. The K5-CreER were developed in our facility and have not previously been tested. Therefore, in this first

experiment we are not only investigating the prostate but we will also run controls to detect the level of recombination. To avoid compromising the prostate studies this characterization will be performed in the skin.

Crosses have been set up for the three existing lines in both directions and so far 35 male animals have been obtained. The DOB of these litters are between 3/13 and 4/26; therefore some of these mice are ready to be analyzed.

Detection of β gal by Immunohistochemistry

As discussed above, we have previously anticipated that the X-gal enzymatic reaction to detect activity will not be adequate to demonstrate that basal cells are positive. The lack of definition of histology performed on thick frozen sections as well as the impossibility of using the confocal microscope are the main problems with the enzymatic reaction. Thus, we developed an immunohistochemistry approach using an antibody against β -galactosidase (Chemicon Cat #AB1211).

After testing several conditions the following technique was established for formaline fixed, paraffin embedded tissues:

Sections were treated using routine protocols (deparaffinization, hydration and blocking of endogenous peroxidases). The best reaction was obtained using antigen retrieval with 10mM Citrate Buffer pH 6.0 for 10 minutes in a microwave oven followed by a 20 minute cool down. After blocking non-specific binding with 10% goat serum, the slides were incubated with primary β -galactosidase polyclonal antibody (Chemicon #AB1211) at a 1:5000 dilution for 30 minutes at room temperature. The slides were then treated with Envision plus labeled polymer, anti-rabbit-HRP (Dako) for 30 minutes at room temperature and the peroxidase reaction developed with DAB monitoring staining development.

As positive controls, we used mouse skin from K5- β gal mice and as a negative control we used wild type siblings from the same colony. The immunostaining of the transgenic samples gave a pattern of staining identical to the Keratin 5 distribution. Wild type controls were negative. We are presently using the technique to stain prostate samples from the K5- β gal animals (we did not have these blocks, we had to process new ones) and to adapt the technique to an immunofluorescence process.

Reportable Outcomes

None

Conclusions

Although we are behind schedule, we have made substantial progress. We now have all the necessary animals to carry out our experiments and we have made the appropriate crosses. We have obtained enough male mice to start the evaluation stage of the project. The only cross that has not been performed is the Probasin-Cre X Rosa 26 but the

Probasin Cre colony is already established and these experiments will begin in the next few weeks. In addition, during this period we have solved a technical problem related to the lack of definition of the X-gal staining by using an immunohistochemistry approach. We expect that during the upcoming period we will be able to stay on schedule and be able to finish the project in a timely manner.

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